

## THE SEXOLOGY OF THE CHIRALLY DIMORPHIC SNAIL SPECIES *AMPHIDROMUS INVERSUS* (GASTROPODA: CAMAENIDAE)

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### INTRODUCTION

Co-occurrence of both enantiomorphs of a chiral body form is found infrequently in nature, rather directional asymmetry (the presence of only one of the two possible enantiomorphs) is the rule (Palmer, 2004). Whereas directional asymmetry is normally genetic, the majority of the few dozen known cases of chiral dimorphism with equal proportions of the two enantiomorphs – “antisymmetry”, as defined by Van Valen (1962) and Palmer (1996, 2005) – are non-inherited and randomized. So far, only two instances of genetic antisymmetry have been described, and both appear to involve reproductive benefits in their maintenance: flower morphology in *Heteranthera multiflora* and other enantiostylous plants, in which antisymmetry promotes outcrossing (Jesson & Barrett, 2002), and mouth orientation in the cichlid fish *Perissodus microlepis*, where disassortative mating exists (Takahashi & Hori, 2008; but see Van Dooren, unpublished data).

Conspicuous genetic chiral dimorphism is also found in snails of the Southeast Asian tree snail genus *Amphidromus* (Laidlaw & Solem, 1961; Asami et al., 1998; Craze et al., 2006; Schilthuizen & Davison, 2005; Schilthuizen et al., 2005, 2007; Sutcharit & Panha, 2006a, b; Sutcharit et al., 2006). Around 36 species are currently included in the subgenus *Amphidromus* s. str., of which at least 30 are chirally dimorphic (Schilthuizen et al., 2005). The other subgenus, *Syndromus*, consisting of approximately 50 species, is entirely sinistral; a species phylogenetically basal to these two clades, *A. glaucolarynx* (formerly placed in *Syndromus*) is, however, also chirally dimorphic, suggesting that chiral dimorphism is the ancestral condition for this genus (Sutcharit et al., 2006). In some species, proportions of dextral and sinistral snails do not deviate significantly from 50/50 proportions (e.g., in *A. martensi* Boettger, 1894; Craze et al., 2006), whereas in others, proportions can be dextral- or sinistral-biased (Sutcharit et al., 2006). However, proportions

of both chiral morphs are almost always substantial, and though not strictly adhering to the definition of antisymmetry, we do not consider the patterns displayed by *Amphidromus* as essentially different from the other known cases of genetic antisymmetry referred to above.

In *Amphidromus inversus* (Müller, 1774), chiral dimorphism appears to enhance mutual fertility. Briefly, this species (and other *Amphidromus* s. str. species; Sutcharit & Panha, 2006b) has a coil in the spermatophore tail, which follows the coiling direction of the snail that produces it (dextral in a dextral individual, sinistral in a sinistral one). After copulation, the tip of this spermatophore coil rests in the recipient individual at the opening of the free oviduct. Since the free oviduct is connected to the spermatophore-receiving organ at an angle, the “fit” is better in dextral-sinistral (inter-chiral) pairs than in sinistral-sinistral or dextral-dextral (intra-chiral) pairs (for more details: Schilthuizen et al., 2007). Modelling (Schilthuizen et al., 2007; Craze, 2009) shows that this situation would, under panmixia, lead to equal proportions for dextral and sinistral morphs (true antisymmetry), but population structure will normally drive a bias for the recessive allele. To all intents and purposes, therefore, chiral dimorphism in *Amphidromus* should be considered a case of genetic antisymmetry.

The evidence for the maintenance of chiral dimorphism in *Amphidromus inversus* derives from studies in a population on the West-Malaysian island of Kapas. This population was discovered by one of us (M.S.) in 1989 and studied intensively since 2003 (Schilthuizen et al., 2005). Later, the same population was described as a new subspecies (*A. i. albulus*) by Sutcharit & Panha (2006a), but since the description is based only on shell colour and shell size, two characters known to be highly variable in *Amphidromus*, we refrain from following their suggestion.

We propose that *A. inversus* might be useful in the future for further studies of mollusc chiral-

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ity and antisymmetry, a field that is likely to become an important component of evolutionary developmental biology (Grande & Patel, 2009). We do so for the following reasons: (1) The population has high density and the snails are easily accessible on low trees in coastal forest. (2) The species' conchology and anatomy have been very carefully described and illustrated by Sutcharit & Panha (2006a, b). (3) Captive or semi-captive individuals are, have been, or will be in culture in the University of Nottingham (Angus Davison), the Edinburgh Zoo (Edwin Blake), the Randers Regnskov Tropical Zoo in Denmark (Asser Øllgaard), the Institute for Biology Leiden (Kees Koops), and in Malaysia (Menno Schilthuisen), and in some (but, admittedly, not all) of these settings, they have fared well, readily copulating, ovipositing and growing. To add to the basis of knowledge for this species, which may become more heavily used in mollusc chirality studies, in this paper we provide a description, based on field observations and field-collected animals only, of copulation and functional anatomy of the genitalia.

#### MATERIALS AND METHODS

We observed parts of the copulation, oviposition, and egg-hatching processes in the field on the West-Malaysian island of Kapas (5°13'00"N; 103°15'30"E; 1.7 × 0.7 km) during five field trips: 29 August–7 September 2003, 26–31 August 2004, 7–18 September 2005, 13–16 October 2005, and 18 May–6 July 2008. Observations were done between 7.00

and 17.00 h while searching snails randomly in coastal and primary forest on the island's west coast and in predefined research plots: sites 1 and 2 from Schilthuisen et al., 2007 (160 × 300 m and 240 × 70 m, respectively) and sites 3, 4, and 5 (previously unpublished, studied in 2008 only, each measuring 50 × 50 m). The positions of mating couples were recorded and, when possible, visited repeatedly throughout the day. We also searched at night on several occasions, roughly between 21.00 and 23.00 h. Twelve copulas in different stages of copulation progress, were fixed in the following way: the mating individuals (and in particular the parts of the genitalia that were extruded) were rapidly and thoroughly sprayed from all directions with electrical component freezing spray, as described in Schilthuisen & Lombaerts (1995) and Craze & Barr (2002), and, while thus frozen, immediately transferred to 70% ethanol. This way, it was assured that the internal genitalia were preserved as exactly as possible in the configuration that they had had during copulation, and the sequence of events during copulation could be reconstructed from them. One copula was carefully separated while in progress, which made both partners expel their spermatophores. The spermatophores were preserved in 70% ethanol. (Please note that we were limited by the copulas that were found in the field, and, though these represent different stages in the copulation process, they do not represent pre-set intervals or are in any way controlled observations.)

Copulas as well as 58 individual adult snails (collected and preserved while not in copula)



FIGS. 1, 2. Copulating pairs of *Amphidromus inversus*, photographed in the field. FIG. 1: Conchologically mature S × S pair; FIG. 2: D × S pair in which at least the sinistral partner is conchologically immature.



were dissected by chipping away the shell(s) with a strong forceps and then dissecting away all non-reproductive organs. In some cases, the genitalia were cleared with clove oil. In all individuals, the bursa copulatrix was removed, opened, and checked for the presence of spermatophore fragments.

Of the egg clutches found in the field, five were hatched in the lab under dark, ambient conditions in bags made of nylon stocking. For the rest, the positions in the field were marked and they were regularly checked until hatching. In addition, three egg clutches were produced at the Edinburgh Zoo (Davison, pers. comm.). For 12 individuals from one of the field-collected egg clutches, total genomic DNA was isolated using a Qiagen DNA extraction kit. A cytochrome oxidase subunit I fragment (mtDNA, COI) was amplified using the universal COI primers of Folmer et al. (1994). Products were cleaned (Qiagen PCR purification kit) and directly sequenced on an ABI377 in both directions using an ABI BigDyePRISM kit.

## RESULTS

Overall, we observed 92 copulating pairs (Fig. 1). Interestingly, some copulating individuals lacked a fully-formed apertural lip, indicating that sexual maturity precedes the end of shell formation (Fig. 2). Courtship is briefly described as follows: two individuals, upon meeting each other on a tree stem or branch, align their shells and then circle one another repeatedly. During this behavior, their heads touch now and then, and they mount each other's shells briefly and repeatedly. Individuals are also seen nibbling each other's palatal apertural shell walls. After approximately 30 minutes, either copulation is achieved or the partners reject one another and continue their separate ways. During the middle of the day (between noon and 15.00 h), mating couples appeared to be engaged in mid-coitus only, and the termination of mating was seen to occur only in the late afternoon and evening (between 15.00 h and 22.00 h). Hence, we estimate (with the caveat that our observation time was largely limited to daytime) that copulations typically last between 5 and 10 hours and are centred around mid-afternoon. Although conically-shelled snails such as *Amphidromus* generally mate non-reciprocally by shell mounting (Asami et al., 1998; Davison et al., 2005; Davison & Mordan, 2007), mating in *Amphidromus inversus* was reciprocal (this could be confirmed by inspecting the parts of the

everted genitalia that were visible in between the copulating partners, and which always consisted of two penis-vagina complexes). Also, contrary to expectation, the snails did not engage in shell-mounting, but in variations on a face-to-face position, although the animals would keep their shells at angles of anything between zero to 180°, depending on their angle of approach and the inclination of the substratum. Some indication was found that the angle between the shells also depends on the coiling direction of the partners: for 39 mating pairs – 24 D × S, 9 S × S, 6 D × D – we measured the angle between the snails' columellae and found that almost all (12 out of 14) intra-chiral pairs held their shells at angles > 45°, whereas of all inter-chiral pairs, the angle in half (12 out of 25) was < 45° (Fisher's exact test,  $P < 0.04$ ). Although no systematic study was made, our field sketches suggest that inter-chiral pairs held the left side of the head of the sinistral partner against the right side of the head of the dextral partner, whereas inter-chiral copulas tended to copulate in a face-to-face manner, with the same side of the head touching in both partners. This is reflected in the difference in angles between their shells while in copula.

Despite these possible differences between intra- and inter-chiral pairs in body orientation during mating, the internal meshing of the genitalia appeared identical in both situations; since the genital organs are long, thin, flexible tubes, twisting to match the partners' chirality appeared no great feat to accomplish. We dissected ten (out of 12 fixed) copulas, leaving their genitalia in situ. This revealed several consecutive stages in copulation. Both partners were always in almost exactly the same stage, hence, below, the actions of only one spermatophore "donor" and one spermatophore "recipient" are described. (We adopt the terminology used by Sutcharit & Panha, 2006b.)

### Stage 1 (one copula; Fig. 3)

The everted penial verge of the donor is inserted into the recipient's vagina. The spermatophore is beginning to form inside the donor's epiphallus and flagellum; simultaneously, sperm is entering the epiphallus from the vas deferens.

### Stage 2 (six copulas; Fig. 4)

The long spermatophore is fully formed, though still rather thin, and its body not yet fully filled with sperm. The epiphallus is much ex-



tended to accommodate it. The tail of the spermatophore remains in the donor's flagellum (its coiled expanded section [CES] remains fixed in the coiled part of the flagellum), and remains empty, while the front part of the spermatophore is being filled with sperm. At the same time, its tip is entering the vagina.

#### Stage 3 (two copulas; Fig. 5)

The tail of the spermatophore remains in the donor's flagellum (the coiled expanded section [CES] of the tail remains fixed in the coiled part of the flagellum), while the front part of the spermatophore is folded into the basal part of the recipient's gametolytic duct. The spermatophore's movement is accomplished by a shortening of the donor's epiphallus. Even as the spermatophore body is entering the gametolytic sac, more sperm continues to be pushed via the vas deferens into the part that is still in the epiphallus.

#### Stage 4 (one copula; Fig. 6)

The tail of the spermatophore is in the process of leaving the donor (the coiled expanded section [CES] of the tail is being pulled out of the coiled part of the flagellum), while the majority of the spermatophore is folded into the recipient's gametolytic duct.

Among the non-copulating individuals, we found no individuals with spermatophores in the male genitalia. This confirms that the spermatophore only begins to be formed upon initiation of copulation and not before. We did, however, find non-copulating individuals with entire or partial spermatophores in the gametolytic duct, which indicated that they had recently mated. This allowed us to recognise three more stages in the process of spermatophore transfer.

#### Stage 5 (two individuals, collected not in copula; Fig. 7)

The entire donor's spermatophore has been folded up into the recipient's gametolytic duct;

the coiled expanded section of the spermatophore tail is jammed in the swollen basis of the gametolytic duct.

#### Stage 6 (one individual, collected not in copula; Fig. 8)

The entire donor's spermatophore is being fragmented in the recipient's gametolytic duct; the coiled expanded section of the spermatophore tail has moved up in the gametolytic duct and no longer closes the entrance to the gametolytic duct.

#### Stage 7 (six individuals, collected not in copula; Fig. 9)

The entire donor's spermatophore is fragmented and the fragments are being transferred to the gametolytic sac.

In all 78 preserved and dissected individuals (both copulating ones and non-copulating ones), we opened the gametolytic sac and in 28 individuals found recognisable fragments of spermatophores in the process of being digested (in cases where at the same time a spermatophore was present in the gametolytic duct, the already strongly-digested fragments in the sac were clearly from a much older copulation). Hence, the final eighth stage in the process of spermatophore transfer is:

#### Stage 8 (28 individuals, collected not in copula; Fig. 10)

The entire spermatophore has been fragmented and is being digested in the gametolytic sac.

We studied one dextral and one sinistral spermatophore. As described previously (Sutcharit & Panha, 2006b; Schilthuisen et al., 2007), the spermatophore is approximately 6 cm long, has five longitudinal, slightly crenulated ridges, and the part that is formed in the epiphallus has a central lumen that is filled with sperm (Fig. 4). The coiled expanded section (CES), which is formed in the tip of the flagellum, contains no

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FIGS. 3–10. Consecutive stages (see text for descriptions of each stage) in copulation and sperm transfer in *Amphidromus inversus*. FIGS. 3–6: Stages 1–4 take place during mating; FIGS. 7–10: Stages 5–8 after coitus has ended (these latter four stages only show the recipient's genitalia). Although fertilization is simultaneously reciprocal, for simplicity, only one donor-recipient genital complex is shown, and parts of the genitalia that are irrelevant at a certain stage are truncated. The spermatophore material is shown in black, the sperm mass is indicated with hatching. All drawings are semi-diagrammatic and (with the exception of FIGS. 3, 6 and 8) composites of multiple dissections (FIG. 4 six dissections, FIG. 5 two dissections, FIG. 7 two dissections, FIG. 9 six dissections, FIG. 10 28 dissections).









FIG. 11. A spermatophore from a sinistral individual of *Amphidromus inversus*; total length approximately 60 mm.

sperm, but has a narrow lumen that opens in a small hole just before the tip. The spermatophore shows several coils of varying tightness. The CES displays three corkscrew-like coils, followed by three much more tightly twisted coils in the neck of the CES, and then three very wide coils in the spermatophore body. The entire structure of the spermatophore is mirror-imaged in individuals of opposite chirality.

We obtained data on clutch size and coiling direction for 38 clutches of eggs and newly-hatched (but yet undispersed) juveniles (Fig. 5a, b; Table 1). Eggs were always found deep in rotting wood, under bark, and in cavities in tree trunks and branches. Five of the clutches were obtained from each of three sinistral and two dextral ovipositing snails, and in four of these, the offspring (which hatched around 40 days after egg laying) were of the same coiling direction as their mothers. Upon hatching,

juveniles had approximately 1.5 whorls. Clutch sizes ranged from 2 to 70 individuals and were always invariant in coiling direction (with one exception; see caption to Table 1). The 12 same-clutch individuals that were sequenced, showed identical COI-sequences, one of which was deposited in GenBank under accession no. FJ472655.

DISCUSSION

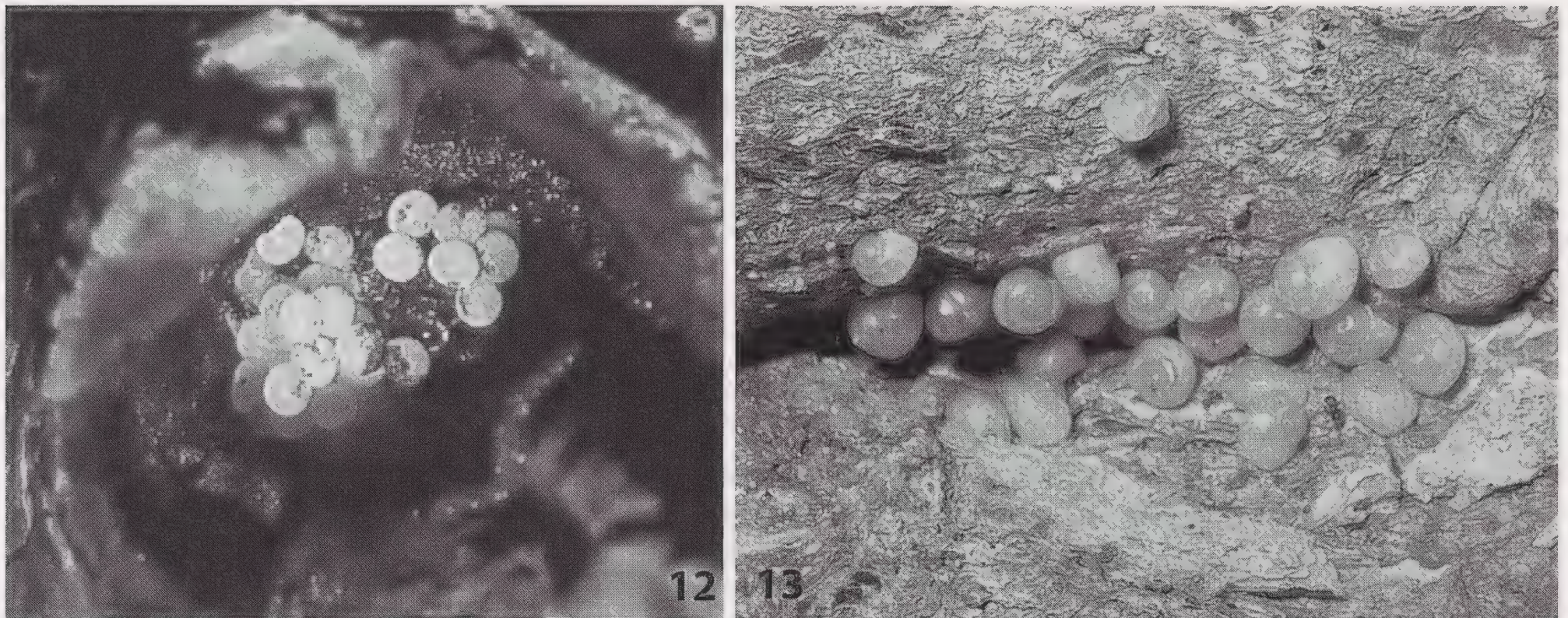
The mating behavior and functional anatomy of the genitalia in *Amphidromus inversus* correspond broadly to those known from other stylommatophoran gastropods (Tompa, 1984). However, some idiosyncrasies are also worth mentioning.

Since there is a tendency for tall-shelled stylommatophoran snails to copulate non-reciprocally and by shell-mounting (Asami et al., 1998; Davison et al., 2005; Davison & Mordan, 2007; but see Jordaens et al., 2009, who showed that the relationship does not hold if basommatophorans are also included), it was surprising to find that *Amphidromus inversus* (and, indeed, other species of the genus as well; Sutcharit et al., 2006) mate face-to-face and reciprocally. In fact, in all copulas studied, the positions of the genitalia and the spermatophore were exactly mirrored in both partners (a similar, exact reciprocity was also observed in *Mastus* by Parmakelis & Mylonas, 2002). In this respect, its mating behavior is similar to that of flat-shelled groups, and indeed, to most other, flat-shelled members of the Camaenidae (Davison et al., 2005; Davison & Mordan, 2007). It may thus constitute a plesiomorphic condition that was retained despite the change in shell shape. Even more surprisingly, the species appears to be unaffected by the generally great impediments to inter-chiral mating. If anything, this species gives preference to inter-chiral over intra-chiral mating (Schilthuizen et al., 2007).

TABLE 1. Data on egg clutches found in the field, in captive animals in Edinburgh Zoo, and ex situ in a garden in Kota Kinabalu. One mixed clutch, presumably the result of two ovipositions in the same locality (Schilthuizen et al., 2007), was treated as two separate clutches.

|                  | Dextral off-spring (total) | Number of clutches | Sinistral off-spring (total) | Number of clutches | Average clutch size | Clutch size standard deviation |
|------------------|----------------------------|--------------------|------------------------------|--------------------|---------------------|--------------------------------|
| Kapas            | 170                        | 10                 | 475                          | 24                 | 19.0                | 12.7                           |
| Edinburgh Zoo    | -                          | -                  | 86                           | 3                  | 28.7                | 13.0                           |
| Garden enclosure | -                          | -                  | 20                           | 1                  | -                   | -                              |





FIGS. 12, 13. Eggs and hatchlings of *Amphidromus inversus*, photographed in the field. FIG. 12: Clutch of recently deposited eggs; FIG. 13: Clutch of recently hatched juveniles, all sinistral; actual diameter of eggs and juveniles, approximately 3 mm.

Neither the mating position nor the interaction of the genitalia during copulation provide obvious clues as to why these obstacles do not play a role in this (and presumably other *Amphidromus* s. str.) species. Sutcharit et al. (2006) reported that in *A. atricallosus*, penes are extended several mm during mating, and that this may facilitate inter-chiral mating. However, in the copulations in *A. inversus* that we observed, the body walls of the partners appeared to be as close together as in the copulation of other snail species. Inter-chiral copulas tended to have their shells aligned, with the left side of the head of the sinistral partner against the right side of the head of the dextral partner. Intra-chiral copulas tended to copulate in a face-to-face manner, with the same side of the head touching in both partners. We suspect that there are, in fact, no intrinsic mechanical reasons why inter-chiral copulation is normally hindered in pulmonates. Rather, the chiral nature of courtship (Lipton & Murray, 1979; Davison et al., 2008) may normally prevent it, since it is adapted to bringing together the genital openings of individuals of the same chirality, and chiral courtship behavior probably has been modified or has become less stereotyped in *Amphidromus* to allow it. Detailed laboratory studies of courtship in *Amphidromus* as compared to chirally monomorphic relatives may clarify this.

We observed several apparently normal copulations in which at least one of the partners was conchologically immature, lacking a fully-formed apertural lip. Clearly, these individuals were reproductively mature. Mating and attempted mating involving conchologically juvenile individuals

has been reported from other pulmonates as well (Tompa, 1984; Webb, 1951).

The sequence of events in spermatophore production, deposition, and digestion, appear to be similar to other stylommatophoran pulmonates. The one copula that we arrested at the very start of mating, shows that the spermatophore is built *de novo* in the epiphallus (the thickest part) and the flagellum (the thinner tail), after the donor's penial verge has been everted and inserted into the recipient's vagina. Since we do not know at what time this couple initiated mating, the time until the beginning of spermatophore formation cannot be determined, but based on what we know from other species, it is likely to be short: in *Helix pomatia*, its formation begins less than a minute after initiation of copulation (Lind, 1973), and in *Arianta arbustorum*, after five minutes (Baminger & Haase, 2001). In *Amphidromus inversus*, sperm starts entering the epiphallus via the vas deferens at the same time as the spermatophore begins forming. That is, the spermatophore is not, as in, for example, Milacidae (Wiktor, 1987), built empty and filled afterwards. Spermatophore formation takes up most of the duration of the copulation, although deposition in the recipient begins before spermatophore formation is complete.

The spermatophore's CES remained in the coiled tip of the flagellum in eight fixed copulas collected around mid-day, although the spermatophore head was already beginning to be pushed into the basal part of the recipient's gametolytic duct by a shortening of the donor's epiphallus. Only in one copula, fixed at the very end of the copulation (early evening), did



the spermatophore begin to move out of the donor's male genitalia to be deposited entirely into the recipient's gametolytic duct. These observations correspond with studies in, for example, *Arianta arbustorum*, where it was also found that spermatophore formation took up over 80% of the mating time (Baminger & Haase, 2001).

Deposition into the gametolytic duct involves the folding of the long, coiled spermatophore into the thick-walled basal part. Initially, the corkscrew-shaped CES remains at the entrance of the gametolytic duct. As reported earlier (Schilthuisen et al., 2007), the tip of the CES (which carries the hole through which sperm is released from the spermatophore) is directed into the free oviduct after an inter-chiral copula, which could enhance sperm uptake in an inter-chiral mating, as compared with an intra-chiral one. The coils may, however, have further implications. Coils in the spermatophore occur in many unrelated land snail taxa. A few of the many examples are *Arianta*, *Mastus*, *Tandonia*, *Milax*, and *Thapsia* (Baminger & Haase, 2001; Parmakelis & Mylonas, 2002; Wiktor, 1987; Winter, 2008) and the springy quality with which they provide the spermatophore might help resist the peristalsis and the closing of the gametolytic organ, allowing more opportunity for spermatozoa to escape from it. Once the entire spermatophore is enclosed in the gametolytic duct, it is fragmented, presumably by a combination of enzymatic weakening and peristalsis in the rather muscular-looking basal part, and the fragments are then transported via the thin stalk to the gametolytic sac for digestion.

Finally, our data on egg clutches and mtDNA data suggest that each egg clutch had been deposited by a single mother snail (with the exception of one clutch, composed of 23 dextral and 2 sinistral juveniles, which may be the result of oviposition by two mother snails in the same place). As in other stylommatophoran pulmonates, the coiling direction of the offspring are identical, which fits the expectation that it is determined by their mother's genotype.

In conclusion, our observations show that intra-chiral as well as inter-chiral copulation and reproduction in the antisymmetric *Amphidromus inversus* do not conspicuously deviate from systems in other stylommatophoran pulmonates, as might have been expected from their characteristic chiral dimorphism. We expect that the obstacles for inter-chiral copulation in directionally asymmetric snails are behavioural, rather than mechanic (see

also Jordaens et al., 2009), and that in *Amphidromus inversus*, these are overcome by small adjustments in the orientation of the partners. The evolution of the coiled spermatophore morphology may prove to be an interesting object of further study. The coils in the spermatophore tip may initially have evolved as a "pressure absorber" to resist the contractions of the gametolytic organ and, as a side-effect, have provided an advantage to inter-chiral copulations because of the better alignment between the donor's spermatophore tip and the recipient's oviduct. As a counter-adaptation, the length of the gametolytic organ may have increased to make the spermatophore lodge further away from the opening (Koene & Schulenburg, 2005), with a resultant further addition of coils in the spermatophore tail. Since *Amphidromus* s. str. species vary greatly in the number and shapes of coils in the CES (Sutcharit & Panha, 2006b), future work may be aimed at understanding spermatophore and gametolytic organ evolution across the subgenus in a phylogenetic framework.

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